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Regulation of Stat3 transcriptional activity by the conserved LPMSP motif for OSM and IL-6 signaling

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Abstract To achieve maximal transcriptional activity in response to gp130 cytokines, Serine-727 (Ser-727) of Stat3 is phosphorylated. Ser-727 resides in the LPMSP motif, the only conserved sequence among the transcription activation domains of several STATs. We show here that in addition to Ser-727, other residues in this LPMSP motif are also required for Stat3 activity in response to cytokine signaling through regulation of Ser-727 phosphorylation and recruitment of the transcription co-activator CBP/p300 to the promoters of Stat3-target genes for transcription activation. Hence, we have demonstrated a critical role for the whole conserved LPMSP motif in JAK-STAT signaling.

Keywords: Stat3; IL-6; Oncostatin M; Transcription; Serine phosphorylation

1. Introduction

Stat3 is one member of the STAT family which is essential for signaling of a wide range of cytokines and growth factors by regulating gene transcription [1]. Stat3 can be activated through tyrosine phosphorylation by a variety of cytokines whose receptors share the gp130 chain, including IL-6, oncostatin M (OSM) and leukemia inhibitory factor (LIF) [2]. Due to its pleiotropic activity, Stat3 plays important roles in many biological processes including oncogenesis, angiogenesis and tumor metastasis [3,4].

The transcriptional activity of Stat3 is mediated by its transcription activation domain (TAD) located in the carboxyl-terminal end of the molecule [5]. The STAT TADs in general can function as independent domains for gene activation by recruiting transcription co-activator CBP/p300 (cAMP-responsive element binding protein [6–10]). The Stat3 TAD contains a conserved sequence motif, LPMSP, also present in the TADs of Stat1 and Stat4 [11]. To achieve maximal transcription activation, the serine residue in the LPMSP motif is also phosphorylated in response to ligand stimulation and required for the maximal transcription activities of Stat1, Stat3 and Stat4 [11,12]. In vivo analyses of S727 of Stat1 and Stat3 using knock-in mouse models showed that S727 plays important physiological roles [13,14]. However, the biochemical function underlying the physiological importance of the phospho-serine and conserved LPMSP motif is not completely understood.

In this report, we demonstrate that other residues in this conserved motif play distinct roles for regulating serine phosphorylation and co-activator recruitment. Our results demonstrate that the whole conserved LPMSP motif, rather than the phospho-S727 alone, is a key regulatory element that controls the maximal transcription activities of STATs.

2. Material and methods

2.1. Cell culture and antibodies

The STAT3$^{-/-}$ mouse embryonic fibroblasts (MEFs) [13] are maintained in DMEM supplemented with 10% bovine calf serum (Hyclone Laboratories Incorporated). Antibodies: anti-phosphoserine-Stat3 and anti-phosphotyrosine-Stat3 (Cell Signaling Technology); anti-Stat3 (Transduction Laboratories); anti-p300 (Santa Cruz Biotechnology). Recombinant human IL-6, IL-6R and mouse OSM were from R&D. Ligand concentrations used were 20 ng/ml IL-6 + 200 ng/ml of soluble IL-6R and 25 ng/ml OSM for lengths of time as indicated in each experiment.

2.2. Chromatin immunoprecipitation (ChIP) assay

ChIP assays were performed as previously described [15] with the following antibodies: 2 μg of anti-Stat3 or 2 μg of anti-p300.

2.3. Plasmid constructions

Stat3 cDNA was amplified by PCR and subcloned into the RcCMV mammalian expression vector (Invitrogen). Specific point mutations in Stat3 were generated with the QuikChange site mutagenesis kit (Stratagene) and confirmed by DNA sequencing.

2.4. Transfection experiments

Transient transfections of Stat3$^{-/-}$ MEF cells were done with the Lipofectamine 2000 method (Invitrogen). Luciferase assays were performed using the Dual-Luciferase Reporter System (Promega). For generation of stable clones, Stat3$^{-/-}$ MEF cells were transfected with the calcium-phosphate transfection kit (Invitrogen) and G418-resistent clones were screened by Western blotting.

2.5. Real-time RT-PCR analyses

Total RNAs were prepared using TRIzol (Invitrogen). Real-Time RT-PCR analysis was performed as previously described [16]. Primer pair sequences are C/EBP delta: 5′ATCGACTTCAGCGCCTACAT, 5′CTAGCGACAGACCCCACAC; c-fos: 5′CCGACCTGCCTGCAAGAT, 5′AATCCAGGGAGGCCACAGA; Tenascin C: 5′TGTGTGCTTCGAAGGCTATG, 5′GCAGACACACTCGTTCTCCA; GAPDH: 5′AGACACCAGTAGACTCCAGG, 5′ACGACCCCTTCATTGACC.

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3. Results

To understand the physiological significance of the conserved LPMSP motif for STAT-mediated transcription activation in response to cytokine signaling, we generated a panel of point mutations in the Stat3 LPMSP motif (Fig. 1A). The various forms of Stat3 mutants were transfected into Stat3$^{-/-}$ MEF cells and phosphorylation of Y705 and S727 were analyzed by Western blotting. OSM induced strong Y705 phosphorylation in all forms of Stat3 (Fig. 1B, middle panel). For S727 phosphorylation, the level of S727 phosphorylation in wildtype Stat3 increased in response to OSM treatment (Fig. 1B, top panel). Similar increases of S727 phosphorylation were detected in the Stat3L724A mutant and the M726A mutant (Fig. 1B, top panel). Mutation of P725A reduced S727 phosphorylation partially (Fig. 1B, top panel) while Stat3P728A had no detectable level of S727 phosphorylation (Fig. 1B, top panel). These results suggest that only the conserved residue P728 is important for OSM-induced Stat3 S727 phosphorylation.

To further determine the effect of these mutations on the transcriptional activity of Stat3, the Stat3$^{-/-}$ MEF cells were transiently transfected with the various forms of Stat3 together with a luciferase reporter containing Stat3 binding sites [13]. Mutations of S727A and P728A reduced Stat3 transcription activity while the Stat3P725A mutant had normal transcription activity compared to the wild type (Fig. 2A). Strikingly, mutations of L724A or M726A significantly reduced the transcription activity of Stat3 in response to OSM (Fig. 2A) even though these mutants had normal S727 phosphorylation (Fig. 1B). The various forms of Stat3 were expressed at similar levels (Fig. 2B). These results demonstrate that in addition to S727, other residues in this LPMSP motif are important for Stat3-mediated gene activation and that S727 phosphorylation alone is not sufficient for Stat3 activity.

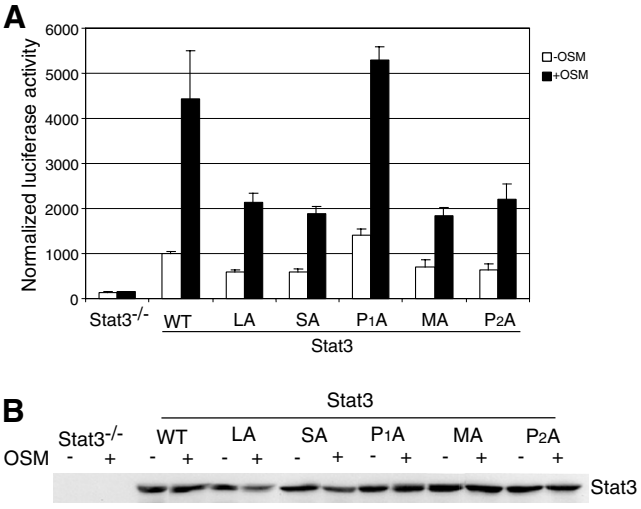

Fig. 2. The conserved LPMSP motif is required for Stat3 transcriptional activity for OSM signaling. (A) The various indicated versions of Stat3 plasmids, a Stat3 luciferase reporter [13] and an internal control Renilla luciferase reporter (Promega) were transiently transfected into Stat3$^{-/-}$ MEFs. Twenty-four hours after transfection, the cells were either left untreated or treated with OSM for 6 h and harvested for luciferase assays. Results shown are luciferase activities normalized against the internal control and the mean + S.D. of four experiments. (B) The lysates used for luciferase assays in (A) were further analyzed by Western blotting for the expression of the various forms of Stat3 mutants.

To understand the molecular mechanism underlying the defect in transcription activity of these Stat3 mutants, particularly those mutants that have normal S727 phosphorylation but lack transcriptional activity such as the L724A and M726A mutants, we studied the ability of the Stat3 mutants to recruit CBP/p300 to the Stat3 target gene c-fos [15]. In

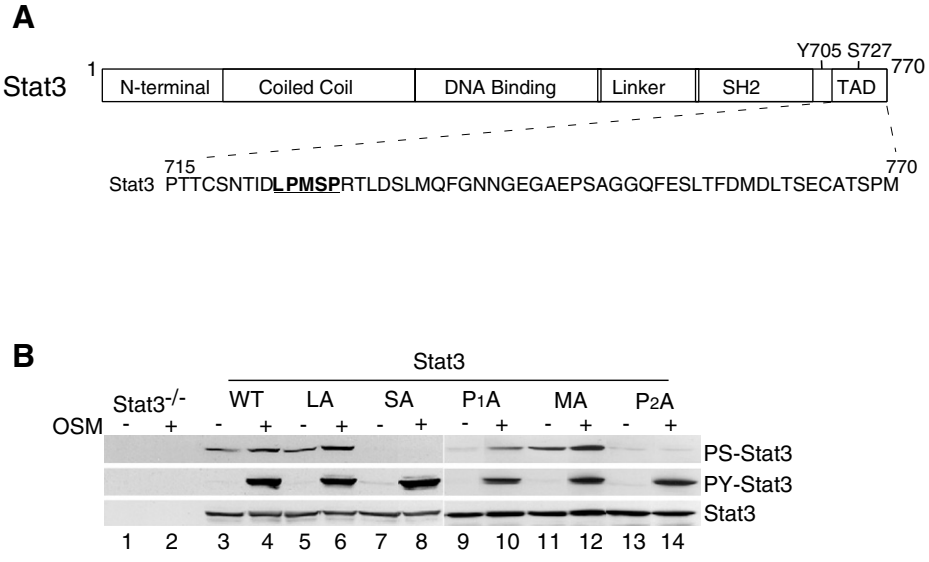

Fig. 1. Requirement of the conserved Proline-728 for Stat3 S727 phosphorylation in response to OSM. (A) A schematic diagram of Stat3 and the sequence of the transcription activation domain. The conserved LPMSP motif is in bold. TAD, transcription activation domain; Y705, tyrosine-705; S727, serine-727. (B) Stat3$^{-/-}$ MEF cells were transiently transfected with Stat3 expression plasmids for 24 h and left untreated or treated with oncostatin M (OSM) for 30 min. Whole cell extracts were analyzed by Western blotting. PS, phospho-S727; PY, phospho-Y705; WT, wild-type; LA, leucine-724 to alanine; SA, serine-727 to alanine; P1A, proline-725 to alanine; MA, methionine-726 to alanine; P2A, proline-728 to alanine.

response to OSM treatment, Stat3 WT and all types of Stat3 mutants bound to the c-fos promoter at similar levels (Fig. 3A). Therefore, mutations in the LPMSP motif do not affect the OSM-induced binding of Stat3 to the c-fos promoter. For CBP/p300 recruitment, OSM could induce a significant increase in the amount of p300 on the c-fos promoter for WT and the P1A mutant (Fig. 3B). All other mutations, particularly remarkable the mutations of L724A and M726A, which had normal serine phosphorylation, caused significant reductions in the amount of CBP recruited to the promoter (Fig. 3B). These results strongly demonstrate that, in addition to the phospho-S727, other residues in the conserved LPMSP motif also plays a critical role in the recruitment of CBP/p300 to Stat3 target gene promoters for cytokine-induced gene activation.

The phenotypes of the Stat3L724A and Stat3M726A mutants are unexpected in that they lack transcription activity despite normal S727 phosphorylation. To see if this is a general defect in response to other gp130 cytokines, the status of S727 phosphorylation and transcription activity of these mutations in response to IL-6 were analyzed with two of the representative mutants of LPMSP, Stat3L724A and Stat3S727A. Similar to the results obtained with OSM stimulation, the Stat3L724A mutant could be phosphorylated on S727 (Fig. 4A, top panel) but had significantly reduced transcription activity in response to IL-6 (Fig. 4B) due to the decreased ability to recruit CBP/p300 (Fig. 4D).

To further demonstrate that the whole LPMSP motif is necessary for Stat3 transcription activity, we analyzed the expression of Stat3 target genes in response to OSM. The expression of three Stat3 target genes, c-fos [15], C/EBP delta [17,18] and Tenascin C [19] was analyzed by Real-Time RT-PCR after

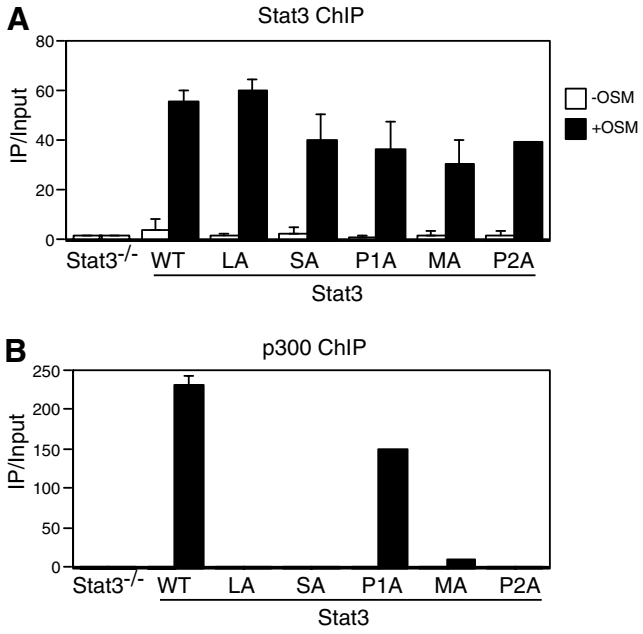

Fig. 3. Stat3-dependent recruitment of the transcription co-activator CBP/p300 to the c-fos promoter in response to OSM. Stat3$^{-/-}$ MEF cells were transiently transfected with the indicated forms of Stat3. Cells were either untreated or treated with OSM for 30 min. Chromatin immunoprecipitation assays (ChIP) were performed with anti-Stat3 (A) or anti-p300 (B). Genomic DNAs were isolated from the IP complexes as well as input lysates and used as templates for PCR with primers specific for the murine c-fos promoter. The ^{32}P-labeled PCR products were separated on acrylamide gels and quantitated by a PhosphorImager. Results shown were the average of two independent experiments and presented as ratios of IP/input.

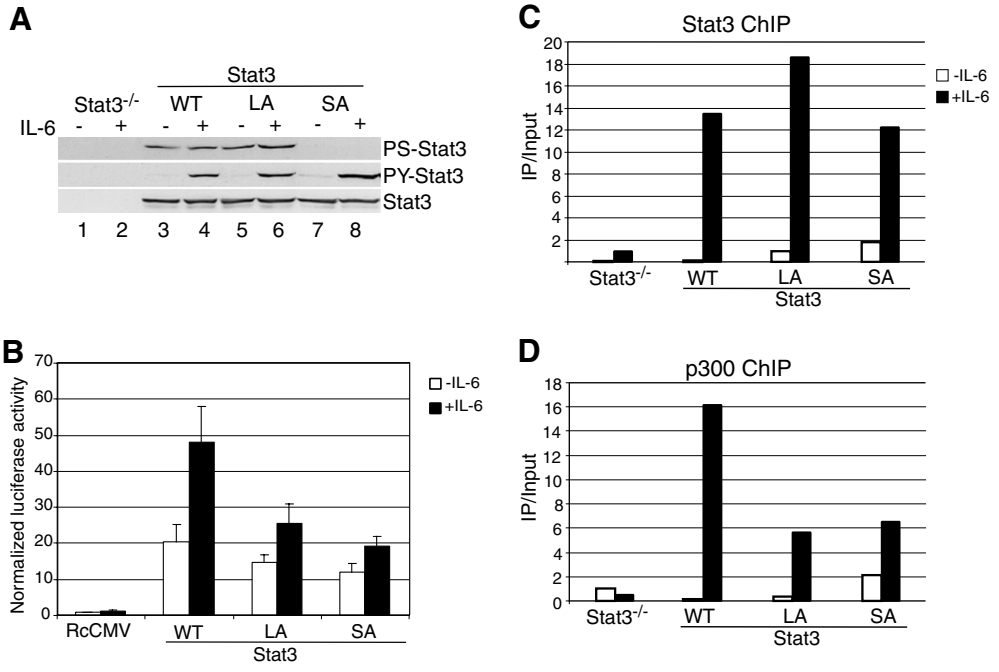

Fig. 4. The conserved leucine-724 residue is required for Stat3 transcription activity for IL-6 signaling. (A) Stat3$^{-/-}$ MEF cells were transfected with Stat3 expression plasmids for 24 h and left untreated or treated with IL-6 for 30 min. Whole cell extracts were analyzed by Western blotting. (B) Luciferase reporter assays were performed as described in Fig. 2. with IL-6 as the ligand. (C–D) Chromatin immunoprecipitation assays (ChIP) were performed with anti-Stat3 (C) or anti-p300 (D) for their binding to the c-fos promoter in response to IL-6 for 30 min. Results of PhosphorImager quantitation were shown as ratios of IP/input for Stat3 (C) and p300 (D).

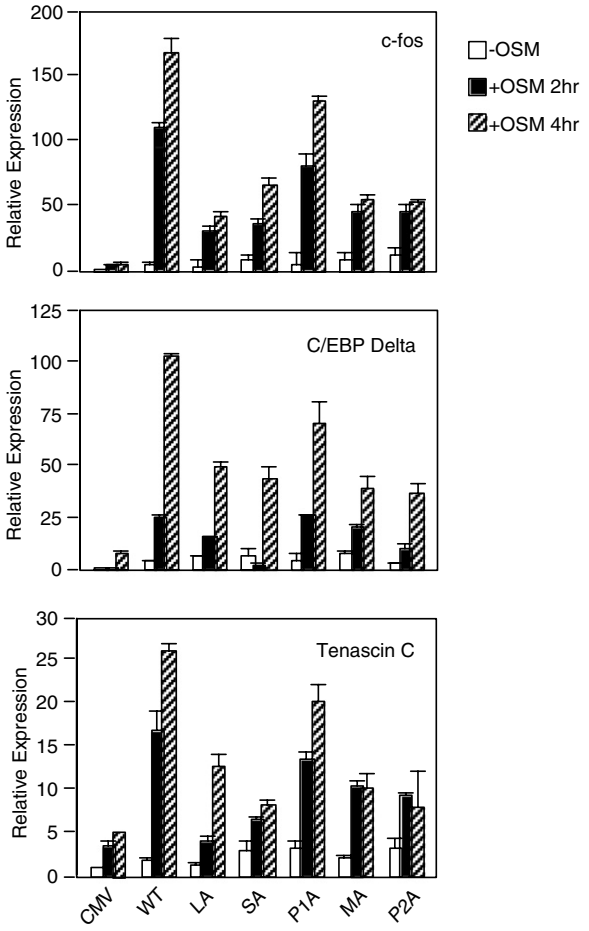

Fig. 5. The complete LPMSP motif is required for activation of Stat3 target genes in response to OSM. Stat3$^{-/-}$ MEFs were transfected with the indicated plasmids for 24 h followed by serum-starvation for 24 h. Cells were either left untreated or treated with OSM for 2 h or 4 h. The expression of Stat3 target genes (c-fos, C/EBP Delta, Tenascin C) and GAPDH were analyzed by Real-Time RT-PCR. Relative expression levels are normalized to GAPDH.

treatment with OSM for 2 h and 4 h. Wild type Stat3 greatly induced all three Stat3 target genes in response to OSM (Fig. 5). Mutation of P725A, which retained normal S727 phosphorylation and ability to recruit CBP/p300, induced gene expression at levels similar to WT Stat3. All other mutations, regardless of their S727 phosphorylation status, caused significant reduction in the expression of all three target genes (Fig. 5). The expression levels of these Stat3 wild type and mutants were equal (data not shown). These results indicate that the whole LPMSP motif, not just serine phosphorylation, is necessary for transcription activation by Stat3 in response to OSM signaling.

4. Discussion

Despite the essential roles that STAT proteins play in many diverse biological processes, the mechanism of transcription activation by STATs is not fully understood. There is no obvious sequence homology between the TADs of STAT proteins except a short sequence motif, LPMSP, found in several STATs that require serine phosphorylation for their maximal activity including Stat1, Stat3 and Stat4 [11,12]. In this report, we show that in addition to the serine phosphorylation, other residues in this LPMSP motif (L724, M726 and P728) are also important for Stat3 transcription activity (Figs. 2A and 3B). Similar results were obtained from mutational analyses of the Stat1 LPMSP motif for interferon signaling [16]. Therefore, it is likely that the whole conserved motif is critical for STATs to mediate transcription activation in response to cytokine signaling.

The regulation of serine phosphorylation of STATs by cytokine signaling is critical to achieve maximal transcription output. Several serine kinases have been shown to phosphorylate Stat1 and Stat3, including the ERK MAPK for Stat3 and the p38 MAPK and CAMKII for Stat1 [20–22]. However, it is not clear how these different serine kinases recognize and bind the STAT TADs as substrates. Here we show that for Stat3, one of the two conserved prolines (P728) is important for S727 phosphorylation while L724 and M726 are not required (Figs. 1B and 4A). In contrast, for IFN-γ-induced Stat1S727 phosphorylation by CaMKII, the conserved L724 is necessary [16]. Together, these results suggest that this conserved motif plays a critical role in presenting the serine residue in different STATs to the distinct serine kinases activated in different cytokine signaling pathways.

Previous studies have demonstrated the importance of serine phosphorylation for the transcription activity of STATs. In this report, we show that while serine phosphorylation is necessary for the maximal activity of STATs, it is not sufficient. In particular, the Stat3L724A and M726A mutants have normal S727 phosphorylation but significantly reduced transcription activity due to its impaired ability to recruit p300 to the c-fos promoter (Figs. 3B and 4D). Therefore, even though some of the mutations of the LPMSP do not affect serine phosphorylation, they have a specific defect in recruiting transcription co-activators for Stat3 target gene activation (Fig. 5). Similar results were obtained from Stat1 mutants [16] suggesting that for optimal interaction between STATs and CBP/p300, the whole LPMSP motif is required. Therefore, it is likely that the complete conserved LPMSP motif is necessary for STAT-mediated transcriptional activation in response to cytokine signaling.

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